Localized Attraction Correlates with Bacterial Adhesion to Glass and Metal Oxide Substra
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Bacterial adhesion to surfaces does not always proceed according to theoretical expectations. Discrepancies are often attributed to surface heterogeneities that provide localized, favorable sites for bacterial attachment. The presence of these favorable deposition sites for bacteria, however, has never been directly measured. Atomic force microscopy (AFM) was used to quantify the distribution of attractive sites on clean substrata. Surfaces of silica and three different metal oxides mapped by adhesion force with regular or colloidal AFM tips showed a heterogeneous distribution of adhesion forces. Adhesion forces were normally distributed based on a colloid probe, but regular tips revealed a proportionately larger number of relatively more adhesive sites. No correlation was found between the average adhesion force (tip or colloid) and macroscopic adhesion tests using five strains of bacteria. However, when AFM tip results were compared to bacterial adhesion data on the basis of only the stickiest sites (the 5% of sites with the largest adhesion force), there was a good correlation of AFM data with adhesion data. These results demonstrate for the first time how overall bacterial adhesion to a surface effectively correlates with a relatively small fraction of highly adhesive sites rather than averaged adhesion force as detected using AFM.

Introduction

Elucidation of the basis for microbial attachment and subsequent colonization of surfaces is integral to a wide number of environmental and technological applications (1–5). The formation of a biofilm that completely covers a surface begins by the adhesion of a small number of bacteria. The relative adhesion of different bacterial strains has been shown to be a function of the physicochemical and thermodynamic properties of both the substrate and the bacterial cell surface, based on analysis of average properties of the surfaces in terms of charge or potential and hydrophobicity. Experimental studies have verified the importance of these properties (6, 7) but have also revealed numerous discrepancies between observations and theoretical expectations. Bacterial deposition is routinely observed even when the estimated electrostatic repulsion between the interacting surfaces produces a potential energy barrier that should be insurmountable for bacteria (6–10). Moreover, cell surface hydrophobicity has been shown to be negligible under many conditions for bacterial adhesion, in contrast to surface thermodynamic predictions (11, 12).

The failure of predictive models is often attributed to the inherent physical and chemical heterogeneities of the solid substrata. Boyd et al. showed that surface roughness on a microscopic scale promoted bacterial retention and concluded that micron-sized surface structures acted as physical barriers to entrap bacteria migrating across the solid surface (13). Surface roughness at the nanometer scale has been shown to increase adhesion (14), but differences in surface roughness on the order of tens of nanometers has been found to be negligible in comparison to other surface properties such as charge (15). Several studies have effectively modeled surfaces with microscopic charge heterogeneity, usually as a patchwork mosaic with individual patches having a uniform surface charge. For such surfaces, particle deposition was shown to be clearly structured, whereas deposition in homogeneous, clean substrata always appeared random (16, 17).

Even ‘ideal’ clean substrata used in controlled laboratory experiments (e.g. glass, metals, and metal oxides) contain intrinsic nanoscale surface heterogeneities. These heterogeneities are due to the complexity of the crystalline structure of solids and their variable chemical composition (18). It has been speculated that surface heterogeneities produce localized, highly favorable sites for bacterial attachment and that these sites control the overall adhesion response of the surfaces (9, 16, 19–21). Indirect evidence from microscopic adhesion studies support the presence of a small proportion of relatively “sticky” sites. Wit and Busscher (20), for example, demonstrated that colloids consistently deposited at the same locations on a substratum surface in repeated deposition experiments using flow cells.

In this study, AFM was used for the direct observation of favorable sites for adhesion on clean solid substrata at the nano- and microscale. Surface areas of four surfaces (glass and metal-oxide coated glasses) were mapped with regard to their adhesion force with regular and colloidal AFM tips. To evaluate the net effect of these surface heterogeneities on microbial adhesion, the attachment of five different strains possessing distinct outermost surface structures (i.e. Escherichia coli D21 and D21Z, Pseudomonas aeruginosa PA01 and PDO300, and Burkholderia cepacia G4) was measured in macroscopic adhesion tests to these different substrata. Considering the ubiquitous nature of microbial adhesion as well as that of silica and metal oxides in soils and many manufactured materials, the results shown here represent a further step toward the understanding of microbial adhesion processes for improved control in industrial and environmental applications.

Materials and Methods

Test Solutions. Experiments were performed in 1 and 100 mM phosphate buffer solution (PBS) at pH 7, and 100 mM PBS at pH 4.5. The 1 mM PBS solution at pH 7 was obtained by mixing 0.026 g of KH2PO4 (Fisher Scientific) and 0.047 g of K2HPO4, respectively. The 100 mM PBS solution was prepared by mixing 0.026 g of KH2PO4, 0.047 g of K2HPO4, respectively, and 0.1 ml of K2HPO4 in demineralized water (MilliQ system, Millipore Corp.). Similarly, the 100 mM PBS solution at pH 4.5 consisted of 1.55 g L−1 of KH2PO4, 5.72 g L−1 of K2HPO4, and 0.023 g L−1 of K3HPO4, respectively.

Solid Substrata and Surface Chemical Composition. Typical glass microscope slides and three different metal oxides-coated glass with TiO2, Al2O3, and Fe2O3 oxide films were used in these experiments. The metal oxides films were produced by chemical vapor deposition techniques, and their thicknesses have been reported to range between 20 and 60 nm (22). Standard glass plates were cleaned by sonication (2 min at 25 °C) in surfactant solution in water (2% RBS35; Environ. Sci. Technol. 2006, 40, 2983–2988

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Pierce Biotechnology), rinsed thoroughly with tap water, dipped in methanol, and again rinsed with demineralized water. Metal oxide-coated glass substrata were cleaned by sonication at 60 °C for 20 min in a glass cleaning solution (Dart 210, Madison Chemical Company, pH = 2.9) and for 10 min at 40 °C in demineralized water. Subsequently, plates were rigorously rinsed with demineralized water and dried with nitrogen gas. After cleaning, all samples were placed in the experimental solution for 2 h to allow equilibrium between the oxide layer and the solution.

The chemical composition of regular and metal oxide-coated glass was determined by X-ray photoelectron spectroscopy using a S-Probe spectrometer (Surface Science Instruments). X-rays (10 kV, 22 mA) at a spot size of 250 μm × 1000 μm (±0.2 μm × 2 μm) were produced using an Al anode. The angle of the photoelectron collector with the nominal to the sample was 35°, corresponding to the surface chemical analysis of the top ∼10 nm. A scan of the overall spectrum in the binding energy range of 1–1100 eV at low resolution (pass energy 150 eV) was followed by scans over a 20 eV binding energy range at high resolution (pass energy 50 eV). The areas under the peaks were used to calculate peak intensities, yielding elemental surface concentration ratios for the chemical elements detected. Results were normalized by the atomic composition based on the detected elements.

**AFM Measurements and Data Analysis.** AFM measurements were performed at room temperature using a Multimode PicoForce System (Veeco Instruments). SiO2, TiO2, Al2O3, and Fe3O4 substrata were mapped in liquid using AFM force volume imaging. Regular silicon nitride AFM tips (Veeco NanoProbe NP-20) having a nominal spring constant of 0.06 Nm−1 and homemade colloid AFM tips were used for the substrata characterization. Topographic height images of the substrata (scan size 15 × 15 μm; 512 lines per sample) were obtained in contact mode using regular silicon nitride AFM tips in demineralized water in order to calculate surface roughness based on root-mean-square heights (RMS; triplicate measurements). Colloid tips were prepared as previously described (23) using latex microspheres (4.5 μm diameter, Polysciences) and tipless silicon nitride cantilevers (Veeco NanoProbeNP-OX). The spring constant of the colloid AFM tips was determined using the thermal tuning method (24). Each colloid AFM tip was tested before and after use for a consistent response when interacting with a clean glass surface. Probes that did not display a consistent behavior were discarded.

Force curves were obtained in 16 × 16 arrays with z-displacements of 100–200 nm at z-scan rates of 10 Hz over scan areas of 15 μm × 15 μm (0.9 μm × 0.9 μm pixel area) and 32 μm × 32 μm (2 μm × 2 μm pixel area) using regular and colloidal AFM tips, respectively. These scan areas were selected so as to ensure that the area probed by the AFM tips did not overlap for two consecutive measurements (contact radius of ∼50 nm and ∼0.5 μm were assumed for regular and colloidal AFM tips, respectively). Five different random locations were studied for each sample and condition. The slope of the retraction force curves in the region where probe and sample were in contact was used to convert the voltage into cantilever deflection. The conversion of deflection into force was carried out as previously described by others (25).

Adhesion maps using retraction force curves were produced by plotting the maximum negative force during tip retraction (Supporting Information Figure S-1) as a function of the x–y location for each force. Adhesion distribution histograms were created from the adhesion maps to quantify the distribution of adhesion forces in terms of a single value. The adhesion distribution histograms associated to the regular tips were characterized in two ways for further comparison to bacterial adhesion data: in terms of an average adhesion force (Favg) and a “local” adhesion force (Fpk). No clear trend in adhesion with surface type was noted when the surfaces were characterized by an average adhesion force (Figure 2a). Only when the localized adhesion force (Fpk) was considered did we see a clear trend of adhesion based only 5% of the greatest adhesion force data was used to calculate the localized surface adhesion force, Fpk. Colloid AFM tip forces were normally distributed and therefore were characterized only on the basis of the average force (Favg).
on surface material (Figure 2b). The localized adhesion forces obtained with the tip and the different solid substrata for all conditions except one (100 mM PBS and pH 4.5) followed the order SiO$_2$ < TiO$_2$ < Al$_2$O$_3$ < Fe$_2$O$_3$. For the one other case, the trend could be described as SiO$_2$ < TiO$_2$ < Al$_2$O$_3$ < Fe$_2$O$_3$. The adhesion force between the colloid probe and the surface was larger than that between the regular tip and the same surface. This was likely due to the much larger surface area sampled by the colloid than the tip. The average adhesion force obtained using colloid tips ($F_{\text{avg}}$) also did not show a consistent trend in adhesion with surface type (Figure 2c). Sometimes there was less adhesion of the colloid to the SiO$_2$ surface than to the metal oxides (PBS, pH 4.5), but in all other cases no clear distinction between the surfaces could be made on the basis of average adhesion forces. This suggests that the colloid tip averaged over too large a surface area to sufficiently characterize the heterogeneity of the surface.

Evaluating Possible Sources of Substrata Heterogeneities. The characteristics of the surfaces were studied in terms of surface roughness and chemical composition (Table 1) so as to investigate the possible causes for the heterogeneous distribution of adhesion forces noted by AFM force measurements. Surface roughness analyses revealed that the surfaces of silica and the three metal oxides are rough at the nanometer level with average values that range from 0.07 nm (SiO$_2$) to 2.37 nm (Fe$_2$O$_3$). Surface chemical composition by XPS further revealed the presence of chemical impurities for nearly all the substrata. Na was found on the SiO$_2$ surface, N on the TiO$_2$ and Fe$_2$O$_3$ surfaces, and C was present on all surfaces as well. Si was observed for the TiO$_2$ surface, but its presence may be due to the influence of the underlying glass surface in XPS scans. The presence of these different elements in the material provides additional evidence for molecular-scale heterogeneity of these surfaces.

### Bacterial Retention on Substrata

To evaluate the potential effect of the surface heterogeneities detected by AFM on microbial adhesion, the adhesive behavior of five
bacterial strains that differed in their outermost surface structures was evaluated in macroscopic adhesion tests to the surfaces. It was found that the type of surface material produced a clear trend in relative adhesion for the five bacterial strains. For all of the solution conditions studied, bacterial retention followed the order SiO₂ < TiO₂ < Al₂O₃ < Fe₂O₃ with regard to the solid substrata (see Figure 3 as an example and Supporting Information Figure S-3). Solution chemistry had a less clear effect than substratum material on bacterial adhesion. Three of the five strains displayed lower retention when solution ionic strength was decreased (100 mM versus 1 mM PBS and pH 7), whereas two of the strains (E. coli D21 and B. cepacia G4) showed the opposite behavior. It was generally observed that a higher number of bacteria remained on the different substrata at the lower pH = 4.5.

Correlation of AFM Data with Macroscopic Bacterial Adhesion. Bacterial retention to solid substrata followed the same trend with substrata type as that found for the localized adhesion force measured between the regular AFM tip and the different substrata, i.e. SiO₂ < TiO₂ < Al₂O₃ < Fe₂O₃. A consistent increase in bacterial retention was observed with respect to the localized adhesion force (F₅%) for each of the five bacterial strains (Figure 4). Only in two cases (B. cepacia G4 and E. coli D21) did the adhesion values obtained under conditions 100 mM PBS and pH 7 deviate from the linearity observed and appeared to be less than those measured under the other solution conditions. The reason for this is not known. A comparison of the average adhesion force obtained with the regular tip or colloid probe (F₉5% and Favg) and bacterial adhesion data did not reveal any significant correlation as no clear trend with surface type was noted when the surfaces were characterized by average adhesion force.

Discussion
Surface heterogeneity on the nano- to microscale has widely been used to explain observed deposition patterns of bacteria in packed bed columns as a function of transport distance (33). In transport models, such heterogeneity is captured by the model with a fast and slow kinetic term to describe relatively adhesive and nonadhesive sites. However, the presence of these two types of sites is a subject of controversy (34, 35). Here we have shown that there is substantial heterogeneity in the relative adhesion of an AFM tip to a surface and that a small percentage of sites appear to correlate with trends observed in the adhesion of bacteria to those surfaces. In addition, as the solution chemistries and surfaces are varied, the tip adhesion force to the surface agrees reasonably well with observed changes in relative adhesion of the bacteria to the surface.

While a small fraction of relatively adhesive sites was measured using the AFM tip, these high-adhesion sites were not evident when using colloid tips as adhesion distribution forces were normally distributed about the mean. This result was surprising as bacteria are closer in size to the colloid probe than they are to the AFM tip. However, in contrast to inert colloidal particles, bacteria are living microorganisms
capable of adapting to their environment in a short period of time. In addition, bacterial surfaces are structurally and chemically more complex and heterogeneous than the surface of synthetic colloidal particles. These structural and chemical heterogeneities arise from the occurrence of fibrils, fimbriae, and other types of surface appendages that have been shown to be responsible for adhesion to a variety of surfaces (36, 37). AFM analysis of bacteria has also shown substantial heterogeneity of bacterial surfaces using phase imaging (38) and single-molecule force imaging (39). Adhesion tests between colloids and bacteria have also provided proof that certain locations of the bacterium, such as one side of E. coli bacteria, are much stickier than other parts of the cell surface (40). Observed deviations from predicted deposition rates in columns have been ascribed to colloid and collector surface heterogeneity (34, 35). As noted by Tufenki and Elimelech (35), the combined influence of both colloid and surface heterogeneities gives rise to the greatest uneven distribution of particle-surface interaction energies. Thus we speculate that bacterial surface heterogeneity is an additional factor that can account for adhesion differences between bacteria and surfaces due to the nature of the localized high-adhesion sites of the bacteria interacting with localized high-adhesion sites on a surface.

Force-imaging of the surface provided good agreement of AFM and bacterial adhesion data based on using a characteristic adhesion value calculated on the basis of only 5% of the most adhesive sites. While this may appear to be too small a portion of the surface to affect the overall behavior of the surface, it is often found that the surface becomes “jammed”, or effectively completely filled, when particles cover the surface at these low values. For example, Liu et al. found jamming limits as low as 3.7% for latex microspheres on glass beads (41). In bacterial transport experiments, surfaces become blocked at values of 4.5% for Pseudomonas putida KT2442 and 5.6% for G4 (42). Even tightly spaced spherical particles on the surface will cover only 54% of the surface due to geometrical constraints (42). Hydrodynamic and colloidal electrostatic repulsion between deposited and approaching particles can lead to smaller surface coverages, effectively filling the space before it is apparently completely occupied (43).

Our findings that a small percentage of the sites on a surface effectively correlate with overall bacterial adhesion surface properties have important implications for modeling of these surfaces. Physicochemical and thermodynamic models that fail to sufficiently characterize bacterial adhesion are based on average properties of the surface, not the localized values that are shown to be important here. In the case of colloid—colloid interactions, it has been shown that such localized charge heterogeneity of these particles is needed to explain particle adhesion for otherwise predicted stable solutions (44). Similarly, our nanoscale adhesion analysis reveals glass and metal oxide surface heterogeneity that is needed to explain bacteria-surface interactions, and therefore models must be modified to incorporate such interactions.

The realization that a small percent of sites dictate the outcome of bacteria-surface adhesion events also has important implications for manufacturing of adhesion-resistant surfaces. Surfaces are typically classified in terms of overall properties based on contact angles and surface charge. Not only will such properties be important in affecting colloidal deposition but also the consistency of the surface will be critical in ensuring resistance of the surface to colloidal deposition. It is clear from data presented here that only a small percentage of the surface will affect the relative adhesion of bacteria to a surface. Once bacteria successfully adhere to a surface, biofilms can rapidly form leading to complete biofouling of the surface. Thus, it will be important to manufacture surfaces with a highly uniform chemistry in order to prevent biofouling.

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Supporting Information Available

An example of a retraction force curve, force maps for all surfaces, and additional adhesion results for bacteria. This material is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited


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